

**Remarks**

Claims 1-19 are pending. Claims 1, 3, 5, 6, 8, 9, 10, 12, 13, 17, 18 and 19 are amended with support as described below. New claims 20, 21 are added with support on page 17 lines 18-24. No new matter is added by these amendments. In view of the following remarks, reconsideration and allowance of the claims are respectfully requested.

**A. Objections**

The disclosure is objected to because it contains an embedded hyperlink and/or other form of browser-executable code. In response to this objection, Applicants have deleted the embedded hyperlink. Withdrawal of this objection in light of this amendment to the specification is respectfully requested.

In regard to the Office's objection that the application does not contain an abstract of the disclosure as required by 37 C.F.R. 1.72(b), Applicants herein submit an abstract of the disclosure on a separate sheet as requested by the Office. Withdrawal of this objection is therefore respectfully requested.

The Office objects to the application for failure to comply with the sequence listing requirements of 37 C.F.R. § 1.821(a)(1) and (a)(2). Specifically, the Office asserts that a statement that the computer readable form and the paper copy of the Sequence Listing are the same is missing. Applicants hereby certify that the information in the computer readable form and the paper copy, both submitted on August 1, 2001, are the same. Withdrawal of this objection is therefore respectfully requested.

The Office objects to the drawings because Figure 2 is described in the Brief Description of Drawings as Figure 2A and 2B, but Figure 2 does not contain an A and B portion. In response, Applicants herein submit a replacement sheet with corrected Figure 2, which contains the required reference to the “2A” and “2B” portions of the figures. Withdrawal of this objection is therefore respectfully requested.

**B. Claim Rejections under 35 U.S.C. § 102**

The Office has attributed two distinct priority dates to Applicants’ claim limitations. Specifically, the Office attributed a priority date of 2/1/2000 for SEQ ID NO: 6-9 and for the citation of specific nucleotide positions of 4550, 4596, 4625, 4626, 4724, 4753, 4754, 4956, 4984, 4985, 5051, 5080 and 5126 of HIV-1 of the HXB2 strain (claims 1, 3-6, 8-10, 12-19). By attributing the priority date of 2/1/2000 to these specific nucleotide positions, the Office has implicitly attributed the priority date of 2/3/1999 for SEQ ID NO: 1-5 and nucleotide positions from 7746-8459 (claims 2, 7 and 11). Therefore, anticipation under 35 U.S.C. § 102, must be determined based on these priority dates.

The Office rejected all pending claims, 1-19, under 35 U.S.C. § 102 as allegedly anticipated by one or more of McDonough, Respass, Pieniazek, Backus EP 0 887 427 A2, Backus 6,001,558, Delaporte and Yang.

For the reasons below, Pieniazek and Yang do not anticipate the claimed range between 4550 and 5126 and Pieniazek and Yang are improperly cited against the claimed range between 7746 and 8459.

**I. Pieniazek and Yang do not anticipate the claimed range between 4550 and 5126**

**a. Pieniazek does not anticipate the claimed range between 4550 and 5126**

Pieniazek is cited by the Office as teaching SEQ ID NO:1-4 of the current application. Because SEQ ID NO:1-4 are all within the claimed range between 7746 and 8459, Pieniazek does not anticipate the disclosed range between 4550 and 5126.

**b. Yang does not anticipate the claimed range between 4550 and 5126**

Yang is also cited by the Office as teaching SEQ ID NO:1-4. Therefore, similar to the above analysis of Pieniazek, Yang does not anticipate the disclosed range between 4550 and 5126.

Because Pieniazek and Yang only disclose primers in the range between 7746 and 8459, their disclosures must be compared to the present application based on the 2/3/1999 priority date attributed to Applicants for these ranges.

**II. Pieniazek and Yang are improperly cited against the claimed range between 7746 and 8459**

**a. Pieniazek is improper 102(b) art against the claimed range between 7746 and 8459**

The rejection of the claimed ranges between 7746 and 8459 based on Pieniazek is improper for the following reasons. First, Pieniazek et al. has a 12/1998 publication date (see copy attached as Exhibit [A]). Second, Pieniazek is cited by the Office as teaching SEQ ID NO: 1-4 of the current application. Because the Office attributed to SEQ ID NO: 1-4 the 2/3/1999 priority date, Pieniazek is not 102(b) art to SEQ ID NO:1-4, which fall in the range between

7746 and 8459. Pieniazek is not 102(b) art because it was published in 12/1998 which is not more than a year before 2/3/1999.

With regard to claimed range between 7746 and 8459, Yang is also improper, because it is a publication by the inventors, and its publication date is less than 1 year prior to the 2/3/99 filing date of the application to which the Office agrees that applicants have priority for this range.

b. Yang is improper 102(a) art against the claimed range between 7746 and 8459

The rejection of the claimed range between 7746 and 8459 based on Yang is improper for the following reasons. The publication date of Yang is 8/1999. Yang is cited for disclosing SEQ ID NO: 1-4, to which the Office has attributed a 2/3/1999 priority date. Therefore, Yang was actually published subsequent to Applicant's invention (as evidenced by the priority date) of the sequences which Yang discloses (SEQ ID NO:1-4), and it is therefore impossible for Yang to be prior art under section 102 (a) for the range between 7746 and 8459.

Because Pieniazek and Yang are improper prior art citations against the claimed range between 7746 and 8459 and because Pieniazek and Yang do not anticipate the claimed range between 4550 and 5126, none of the claims are properly rejected under these two references.

Therefore, Applicants respectfully request withdrawal of the rejections based on these references.

**III. Delaporte and Backus EP 0 887 427 A2 are improper 102 (e) art**

a. Delaporte is improper 102(e) art

The Office rejects Claims 1, 2, 4, 6, 10 and 15 under 35 U.S.C. 102(e) as being anticipated by Delaporte. This rejection is improper because the appropriate 102 (e) priority date for Delaporte is April 3, 2000, which is subsequent to both the 2/3/1999 and 2/1/2000 priority dates attributed to the present application. For Delaporte to be 102(e) prior art to the present application, the reference would need to be awarded a 102 (e) priority date of its related international application No. PCT/EP98/04522, filed on July 20, 1998. This is not, however, Delaporte's proper 102 (e) date. MPEP § 2136.03 II.(C)(3) outlines how to award a priority date under 102 (e) for U.S. application publications that claim benefit under 35 U.S.C. 120 or 365 (c) of an international application filed prior to November 29, 2000. In such cases, the reference is applied under the provisions of 102 (e) prior to the AIPA amendments.

Delaporte is a U.S. application publication that claims benefit, through 35 U.S.C. § 120 to application No. 09/462,917, and through 35 U.S.C. § 365 to an international application filed prior to November 29, 2000. Because of these facts, Delaporte's proper 102 (e) date is the actual filing date of the later-filed U.S. application that claimed benefit of the earlier application. See MPEP § 2136.03 II (C)(3); See also MPEP § 706.02(a)(II). Thus, the proper 102 (e) date for Delaporte is the actual filing date of application No. 09/462,917, which is April 3, 2000. Because the proper 102 (e) date for Delaporte is April 3, 2000, it is not prior art to Applicants since April 3, 2000 is subsequent to even the latest acknowledged priority date..

b. Backus EP 0 887 427 A2 is improper 102 (e) art

The Office rejects Claims 1, 3-6, 8-10 and 17-19 under 35 U.S.C. 102(e) as being anticipated by Backus EP 0 877 427 A2. Backus EP 0 887 427 A2 is not proper 102 (e) art

because it does not fall within the statutory requirements of 102(e). References based on international applications that were filed prior to November 29, 2000 are subjected to the pre-AIPA version of 35 U.S.C. 102(e). See MPEP § 706.02(a). The MPEP further states that, "No international filing dates prior to November 29, 2000 may be relied on as prior art under 35 U.S.C. 102(e) in accordance with the last sentence of the effective date provisions of Pub. L. 107-273." Moreover, even under the current version of 102(e), Backus EP 0 887 427 A2 would not be proper 102(e) art because it was not either filed in the U.S. or an international application.

Because Delaporte and Backus EP 0 887 427 A2 are improper citations of prior art, Applicants respectfully request withdrawal of the rejections based on these references.

Claims 1, 3-6, 8-10, 12 and 17-19 are also rejected under one or more of McDonough, Respass and Backus 6,001,558.

**IV. Amended claims 1, 3, 5, 6, 8-10, 12 and 17-19 are not anticipated by McDonough, Respass or Backus 6,001,558**

Claims 1, 3, 5, 6, 8-10, 12 and 17-19 have been amended herein to overcome the Office's 35 U.S.C. § 102 rejections based on Respass, McDonough and Backus 6,001,558. Claim 4, although not amended, depends from amended Claim 1.

McDonough, Respass and Backus 6,001,558 are all cited by the Office for disclosing oligonucleotide primers to detect HIV-1. Specifically, the Office Action states that McDonough teaches oligonucleotide primers chosen from regions corresponding to nucleotides 4756-4778, 4835-4857 and 4952-4969 from HIV-1 HXB2. Respass is cited as disclosing a primer pair that amplifies sequences from 4750-4990 of HIV-1 HXB2. Backus 6,001,558 is cited as disclosing a

sequence with 100% homology with base pairs 3 to 31 of Applicants' SEQ ID NO: 9. Further, Backus is cited as disclosing a primer that binds between 4897 to 4924 of HIV-1 HXB2. The Office incorrectly asserts that primers for the env region of HIV-1 are disclosed by Backus. Backus only cites an env primer for HIV-2.

Combining the rejections based on McDonough, Respass and Backus 6,001,558, it is evident that the Office considers that the following HIV-1 ranges and sequences are disclosed:  
4750-4990, 4756-4778, 4835-4857, 4952-4969, 4897-4924 and SEQ ID NO: 9.

To overcome these rejections, Applicants have amended the Claims by excluding oligonucleotides that selectively hybridize to a region of HIV-1 HXB2 including 4754-4984. Moreover, Applicants have also removed SEQ ID NO: 9 from all Claims rejected under McDonough, Respass and Backus 6,001,558. Applicants have support for excluding this range, since this is a range specifically disclosed and was claimed by Applicants. MPEP section 2173.05(i) states that "The current view of the courts is that there is nothing inherently ambiguous or uncertain about a negative limitation. So long as the boundaries of the patent protection sought are set forth definitely, albeit negatively, the claim complies with the requirements of 35 U.S.C. 112, second paragraph. Some older cases were critical of negative limitations because they tended to define the invention in terms of what it was not, rather than pointing out the invention...Any negative limitation or exclusionary proviso must have basis in the original disclosure. *If alternative elements are positively recited in the specification, they may be explicitly excluded in the claims.*" (Emphasis added.)

Moreover, in *In re Johnson*, 558 F.2d 1008, 194 USPQ 187 (CCPA 1977), the CCPA held that a claim to a genus with the limitation of a negative proviso that did not appear in the original specification complied with the written description requirement (for the purpose of establishing benefit of an earlier filing date to overcome a prior art rejection based on applicants' earlier foreign-filed patent). The negative proviso, which was inserted to avoid having the claim read on a lost interference count, literally excluded more than the two species disclosed in the application (and the full scope of the negative proviso was clearly understood and acknowledged by the court; *ibid* at Note 12). The court stated:

The notion that one who fully discloses and teaches those skilled in the art how to make and use a genus and numerous species there within, has somehow failed to disclose, and teach those skilled in the art how to make and use, that genus minus two of those species, and has thus failed to satisfy the requirements of § 112, first paragraph, appears to result in a hypertechnical application of legalistic prose relating to that provision of the statute.

*Ibid* at 1019.

The holding in *Johnson* was affirmed by the court in *In re Driscoll*, 562 F.2d 1245, 1250, 195 USPQ 434 (CCPA 1977), which observed (with regard to the court's reversal, in *Johnson*, of the Patent Office's refusal to grant Johnson's application the benefit of an earlier filing date, based on an alleged lack of written description, in the original application, for the negative proviso):



In reversing the rejection, the court there observed that the applicants were merely excising the invention of another, to which they were not entitled, rather than creating an artificial subgenus or claiming new matter.

*Ibid* at 1250.

Therefore, there is no requirement in the patent law that the specification must state that something can be excluded in order for there to be support for that exclusion. The only requirement is that what is to be excluded must have been disclosed. Because the application discloses “4754-4984,” the recitation of a primer “wherein the oligonucleotide does not selectively hybridize to a region of the HXB2 strain between 4754-4984”, does not constitute new matter.

Applicants believe that the amended Claims overcome the rejections for the following reasons.

First, the largest range cited by the Office is disclosed by Respass. The Office states that Respass discloses a primer pair that amplifies the sequence from 4750-4990. The actual primer pair that amplifies the 4750-4990 range consists of two individual primers. The first selectively hybridizes between 4750-4782, and the second hybridizes between 4957-4990. These are the only relevant primers disclosed by Respass. To overcome anticipation by these primers, Applicants must not claim these individual primers. By excluding primers that hybridize to 4754-4984, the amended claims exclude the primers taught by Respass, since both primers of

Respass hybridize at least in this range. By excluding this range, the claims do not read on the specific primer of Respass.

Second, because the exclusion of primers that hybridize to 4754-4984 excludes all the ranges cited by the Office as disclosed by McDonough (4756-4778, 4835-4857 and 4952-4969).

Third, Applicants have removed SEQ ID NO: 9 (4956-4984) from the claims by excluding 4754-4984. By excluding this sequence, Applicants claims are not anticipated by Backus' SEQ ID NO: 8 (base pairs 3 to 31 of Applicants' SEQ ID NO: 9).

Applicants believe that the amendments overcome the 35 U.S.C. § 102 rejections over Respass, McDonough and Backus 6,001,558. Applicants also note that none of the cited references suggests the claimed primers. Reconsideration of the claims is respectfully requested.

In summary, all of the rejections recited under any section of 102 are either improper for the reasons cited above, or are overcome as described above.

**C. Claim Rejections under 35 U.S.C. § 112, Second Paragraph**

Claims 1-19 are rejected under 35 U.S.C. § 112, second paragraph for allegedly being indefinite and failing to particularly point out the claimed subject matter. The Office Action states that Claims 1-19 are allegedly vague for reciting that the oligonucleotide primer "selectively hybridizes." The Office Action continues that because the specification does not provide a standard for ascertaining the requisite degree, the metes and bounds of this claim cannot be established. With respect to these concerns the answer is that Applicants have provided a standard for ascertaining the requisite degree for selectively hybridizing on page 9 of the specification from lines 20-26. Applicants believe that the definition of "selective" and

“selectively” on page 9 make the Claims clear as written. The standard is clear in that requires specificity of hybridization that allows for determination of HIV-1 infection.

Unless the Office is aware of a specific basis to believe that the terms “selectively” and “hybridize” would not be understood by the skilled person to have their usual meaning, there is no basis for the present rejection. There are numerous examples in the relevant scientific literature of the terms “selective” and “hybridize” in the context of oligonucleotide hybridization and amplification (primers) (See attached abstracts, Exhibits B-I). This evidence establishes that the skilled person knows the scope and meaning conveyed by these terms in reference to primers and their use in amplification. Applicants’ use of these terms is consistent with that accepted in the art. Since the Office has provided no basis to contradict this substantial evidence that the term is clear, the rejection should be withdrawn. Applicant respectfully requests withdrawal of this rejection.

Claims 5, 9, 13, 15 and 19 are rejected under 35 U.S.C. § 112, second paragraph as allegedly unclear for reciting primers with nucleic acids with conservative substitutions thereof. With respect to this rejection Applicants have amended Claims 5, 9, 13, 15 and 19 by deleting the phrase “conservative substitutions thereof.” By deleting “conservative substitutions thereof” from the claims, Applicants believe that this rejection is overcome.

The Office rejected Claim 17 under 35 U.S.C. § 112, second paragraph for reciting that the first primer comprises the nucleotide sequences of SEQ ID 5-9. The Office Action states that it is unclear how a single primer can be comprised of 5 divergent nucleic acid sequences. With respect to this rejection, Claim 17 has been amended to recite wherein the first primer comprises

a nucleotide sequence “selected from the group consisting of.” Applicants believe that this amendment makes it clear that a single primer is not comprised of 5 divergent nucleic acid molecules but rather is selected from the group of nucleic acid molecules.

**D. Rejections under 35 U.S.C. § 112, first paragraph**

Claims 1-3, 7-8, 10 and 18 were rejected under 35 U.S.C. § 112, first paragraph as allegedly containing subject matter not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention.

The Office Action states that in the instant case applicants do not disclose any primers that will bind to a highly conserved region between nucleotides 4550-5126 or 7746-8459. Further, the Office Action states that the disclosure of no examples in a genus would not represent to the skilled artisan a representative number of species sufficient to show applicants were in possession of the claimed genus. In regard to these statements, Applicants respectfully disagree.

Applicants disclose nine specific sequences, represented by SEQ ID NOs: 1-9. Of these nine specifically identified sequences, the first 5 selectively hybridize to HIV-1 between nucleotide positions between 7746-8459 (SEQ ID NO:1 between 7789-7816; SEQ ID NO:2 between 8347-8374; SEQ ID NO:3 between 7850-7879; SEQ ID NO:4 between 8281-8310; SEQ ID NO:5 between 8265-8294), and the second 4 selectively hybridize between nucleotide positions 4550-5126 (SEQ ID NO:6 between 4596-4625; SEQ ID NO:7 between 5051-5080; SEQ ID NO:8 between 4724-4753; SEQ ID NO:9 between 4956-4984). Based on these

disclosed sequences, Applicants provide description of primers in the claimed upper and lower ranges. The exemplified primers from the lower range are representative of the lower range because they cover most of the lower recited range. The exemplified primers from the upper range are representative primers of the upper range because they cover most of the upper recited range. Thus, the claimed genus of primers is exemplified by representative species in satisfaction of the guidelines for written description.

Thus, applicants do provide sufficient description of a representative number of species by actual reduction to practice, and thereby disclose examples of the claimed genus to represent to the skilled artisan a representative number of species to show Applicants were in possession of the claimed genus. Moreover, Applicants have shown possession of the claimed genus for the following additional reasons.

The rejection as written suggests that the Office has not given any meaning to the term “selectively.” This seems to have created a misapprehension of the scope of what HIV-1 nucleic acids the primer will hybridize to. Although not acknowledged by the Office, the Claims specify selective hybridization to nucleic acid of the HXB2 strain. This reference sequence provides structural definition to the sequence of the primers Applicants have provided the HXB2 target sequence in the specification as GenBank accession K03455. Moreover, Applicants note that “selective” and “selectively” are defined clearly in the specification.

Also, selective hybridization of primers to target nucleic acids is predictable. There is no scientific support for the Office’s assertion that nucleic acid hybridization is unpredictable. In fact, Watson-Crick base pairing, which is the basis for hybridization in the present claims, is one

of the most predictable phenomena in molecular biology. It is the foundation of numerous universally accepted and used molecular biology techniques, including PCR, Northern blotting and Southern blotting. Thus, alleged unpredictability is not a legitimate reason for the present rejection.

Since the ranges are clearly defined, since what is a conserved region is readily envisioned by the skilled person, and since the sequence of the claimed primer is limited by what will hybridize selectively to HXB2, the genus is sufficiently defined to be within Applicants' possession. Therefore, given that Applicants provided the sequence of a specific exemplary target HXB2, defined "selective" and "selectively," and provided working examples from within the genus, and given the predictable nature of selective hybridization, the skilled artisan would understand that Applicants are in possession of the claimed genus.

Credit Card Payment Form PTO-2038 authorizing payment in the amount of \$ 110.00 (representing the fee for a one-month extension of time) is enclosed. This amount is believed to be correct. However, the Commissioner is hereby authorized to charge any additional fees which may be required or to credit any overpayment to Deposit Account No. 14-0629.

**ATTORNEY DOCKET NO. 14114.0346U2**  
**Application No. 09/890,551**

Respectfully submitted,

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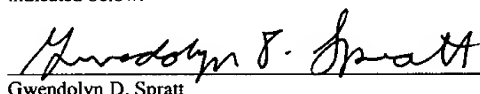
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Gwendolyn D. Spratt

2-2-04  
Date

## Appendix

### ABSTRACT

Reagents and assays for detecting HIV-1 groups M and O and optionally HIV-1 group N and SIVcpz are provided. The reagents are nucleic acid primers for the hybridization to, amplification and subsequent detection of HIV-1 groups M, N and O and SIVcpz in a biological sample. The primers are oligonucleotides that selectively hybridize to the highly conserved regions of the *env* and *pol* regions of HIV-1. The assays employ the primers for HIV-1 nucleic acid amplification using amplification techniques such as reverse transcription and the polymerase chain reaction. The assays are useful for diagnosing an HIV-1 group M, HIV-1 group N, or an HIV-1 group O infection in a patient. Due to the high sensitivity of the assays, small concentrations of HIV in a biological sample can be detected, thereby allowing diagnosis at an early stage of infection. The assays are also useful for detecting HIV-1 contamination in a biological fluid such as blood. The assays are qualitative or quantitative and are therefore useful for viral load determinations of HIV-1 groups M, N or O in a patient undergoing treatment for HIV-1 infection. Viral load determinations can be used to monitor the progress of the treatment regimen or the development of drug resistance and can be used to predict disease progression.



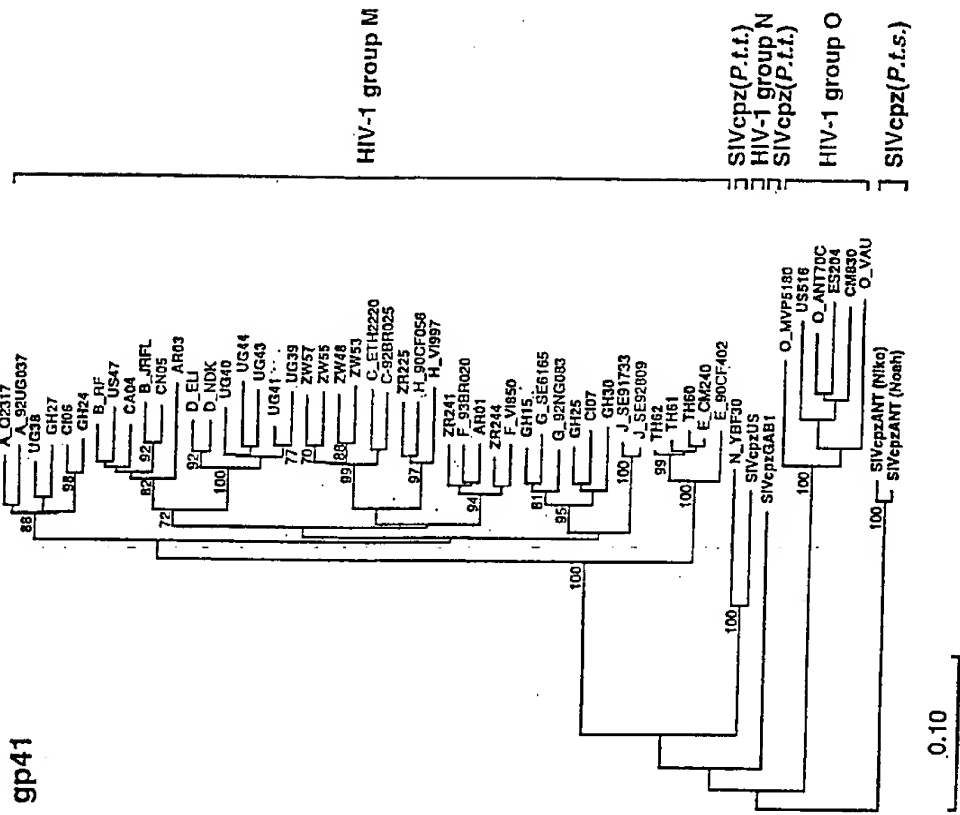


FIG. 2B

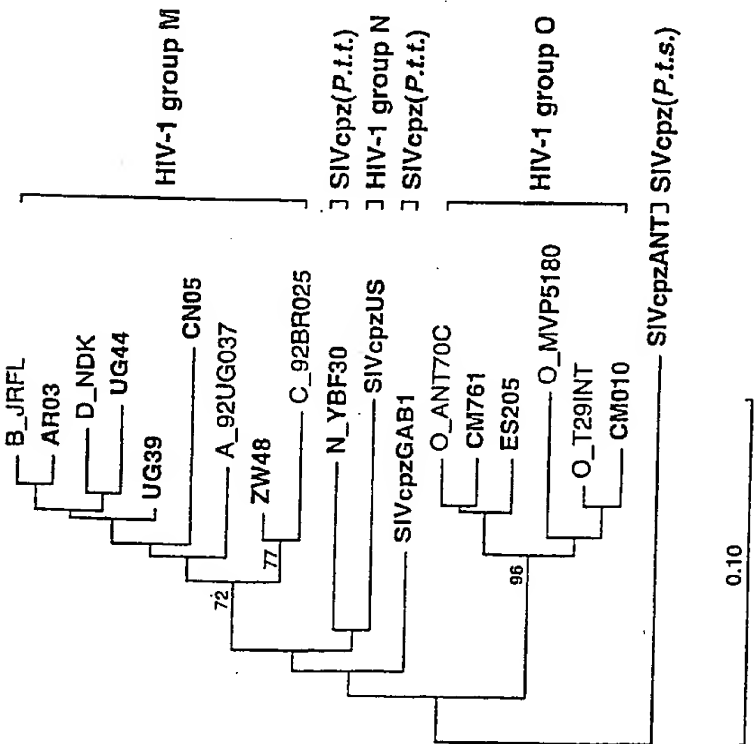


FIG. 2A

## Exhibit A

### Phylogenetic Analysis of gp41

# Phylogenetic analysis of gp41 envelope of HIV-1 groups M, N, and O strains provides an alternate region for subtype determination

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Human immunodeficiency virus type 1 (HIV-1) is characterized by an unusually high degree of genetic variability in vivo. Analysis of HIV-1 *env* sequences of virus strains from different geographic locales has revealed that HIV-1 can be divided into three main groups, M (major), O (outlier), and N (new). HIV-1 group M has been further subdivided into genetically equidistant clusters of HIV-1 *env* genes, comprising subtypes A-J [1, 2]. Some HIV-1 variants reported as "uncertain" or "unclassifiable" (category U) may represent either a new subtype or a recombinant sequence and their designation may be clarified as more reference sequences become available [2]. Likewise detailed molecular analysis of many group O strains have not revealed any specific clustering into subtypes [3]. More recently, a new variant of HIV-1, termed group N, has been discovered in Cameroon [4]. These genotypic analyses have resulted in better understanding of the molecular epidemiology of HIV-1 and improved tracking of the epidemic, in addition to defining the geographic distribution and transmission patterns of various HIV-1 group M subtypes [2].

While full-length genomic analyses are needed for new subtype designation and to identify recombinant viral genomes [2], they are not suitable for large molecular epidemiologic studies due to labor and time expense. Thus the shorter fragments of the genome still remain the primary tool used to monitor HIV-1 genetic diversity worldwide. Since the *env* sequences provide information on all known and potentially new subtypes circulating in a given geographic area, the *env* region remains the principal target for HIV-1 subtyping to assist in continued identification of old and new variants. The most commonly used procedure for HIV-1 subtyping has relied on analysis of the C2-V3 segment of *env*. While most PCR-derived sequences contain a suboptimal length for phylogenetic analysis, a comparative analysis with larger *env* sequences has revealed that limited V3 region sequences can generally serve as a reliable basis for subtype determination [2, 5]. However, because of the broad heterogeneity within the C2-V3 domain of HIV-1 group M viruses and the constant nucleotide changes in this region over time [5], a plethora of different sets of primers are needed to maximize the efficiency of PCR amplification and sequencing worldwide. Moreover, creating the DNA sequence alignments from clinical specimens may be extremely difficult because of tremendous nucleotide variations due to deletions and insertions in this region. In addition, different sets of the C2-V3 primers are needed to generate data for group O and N viruses.

To circumvent these practical problems, we sought to determine if a highly stable transmembrane *env*-gp41 could serve as an alternate region for a global phylogenetic analysis of HIV-1 subtypes. Our initial approach was to determine whether HIV-1 gp41 and C2-V3 sequences would cluster into the same phylogenetic subtypes. A parallel phylogenetic analysis of the C2-V3 and gp41 sequences was done using group M, group N and group O reference sequences from full length genomes, as well as commonly used marker sequences of subtypes A-H and chimpanzee viral sequences of CPZ-ANT and CPZ-GAB strains [6, 7]. The results of the phylogenetic analysis demonstrated an agreement between phylogenetic clustering of gp41 and C2-V3 HIV-1 sequences into group M subtypes A-H viruses, group N, and group O by using both the maximum-likelihood and neighbor-joining methods (Fig. 1). In the neighbor-joining method, bootstrap values at branch nodes connected with subtypes range from 95% to 100% for both C2-V3 and gp41 trees. More importantly, while there were differences in the overall topologies of the phylogenetic C2-V3 and gp41 trees, the subtype assignment did not change in any case. Likewise, a comparison of phylogenetic analysis of *env* C2-V3 versus V3-V5 fragments showed differences in tree topology, although the subtype designations remain unchanged [10]. Thus, based on the remarkably similar phylogenetic clustering of C2-V3 and the gp41 region, we conclude

that the global diversity of the gp41 region is sufficient to provide a reliable marker for phylogenetic clustering of HIV-1 subtypes.

Despite the nucleotide divergence within the gp41 region, which allowed phylogenetic analysis for subtype determination, some fragments of the gp41 region showed enough conservation to permit the design of consensus PCR primers. Thus, we designed one set of *env* gp41 primers, namely gp41M/O, for a nested PCR amplification and sequence analysis of group M, N, and O viruses [2]. The primers are:

Name	Direction	Primer Sequence	Genomic Position*
gp40F1	forward	5' TCTTAGGAGCAGCAGGAAGCACTATGGG	7789–7816
gp41R1	reverse	5' AACGACAAAGGTGAGTATCCCTGCCTAA	8347–8374
<b>Nested PCR</b>			
gp46F2	forward	5' ACAATTATTGTCTGGTATAGTGCAACAGCA	7850–7879
gp47R2	reverse	5' TTAAACCTATCAAGCCTCCTACTATCATT	8281–8310

\*Position by alignment to HXB2, GenBank accession number K03455.

See HXB2 numbering system at <http://hiv-web.lanl.gov/NUM-HXB2/HXB2.MAIN.html>

The primer sequences are highly conserved for group M, group N, and group O sequences (<http://hiv-web.lanl.gov/ALIGN-99/subtype-alignments.html>). The PCR conditions included denaturation at 94° C for 2 min, followed by 35 cycles of denaturation at 94° C for 30 sec, annealing at 50° C for 30 sec, and extension at 72° C for 60 sec, with a final extension at 72° C for 5 min. The PCR amplification conditions using DNA-PCR and RT-PCR assays for peripheral blood mononuclear cells (PBMC) and plasma, respectively, have been described elsewhere [11]. A fragment of about 460 bp spanning approximately 40% of the gp41 region, which includes the immunodominant region, was successfully amplified from as few as 1 to 5 copies of viral DNA from nearly full-length HIV-1 clones representing group M subtypes A–H. The assay is highly sensitive in detecting plasma viral RNA from HIV-1 strains of diverse geographic origins representing different subtypes of HIV-1 group M as well as HIV-1 group O. Of the 253 group M plasma specimens (subtypes A = 68, B = 71, C = 19, D = 27, E = 23, F = 33, and G = 12), 250 (99%) were amplified using the gp41M/O primer set. More importantly, all 32 (100%) group O plasma samples were also amplified with these primers [11]. *In vitro* spiking experiments further revealed that the assay could detect as few as 10 copies of viral RNA/mL of plasma and gave positive signals in selected HIV-1 seropositive plasma, with viral copy numbers below the detection limits of all commercially available viral load assays. Moreover, the gp41M/O primer set amplified viral DNA of the group N YBF30 strain indicating the potential of these primers to amplify new divergent viruses. Additionally, the gp41M/O primers were highly specific for HIV-1; no PCR amplification of HIV-2, SIVs, and SIV-cpz strains was observed. Therefore, our findings indicated that the universal gp41M/O primer set that we designed was indeed highly specific, sensitive, and efficient in a PCR amplification of the gp41 region, regardless of the genotypes and geographic origin of the viruses. Finally, we have performed phylogenetic analysis of gp41 sequences from more than 500 group M and 32 group O specimens that were successfully amplified from PBMCs and plasma as a part of various ongoing studies worldwide. The representative group M subtype A–G and group O sequences from selected geographic regions (Thailand, Egypt, Uganda, Ghana, India, Zimbabwe, Argentina, and Cameroon) are shown in Fig. 2. As expected, sequences from Thailand specimens clustered on a distinct branch within the subtype B, as had previously been shown for C2–V3 sequences. One of four unclassifiable variants had variable positions in the tree topology depending on the phylogenetic method used (Fig. 2). Analysis of other parts of viral genomes from these four variants, including the entire *env* region, is now underway in order to classify them into either potential new subtypes or recombinants along the *env* gene. Taken together, these studies indicate that gp41 provides a simple and practical region for subtype determination of group M HIV-1.

In conclusion, we have found that genetic analysis of PCR-amplified HIV-1 gp41 sequences provides a powerful approach for identifying groups M, N, and O viruses. Furthermore, group M

## Phylogenetic Analysis of gp41

viruses can be phylogenetically classified into at least seven subtypes, the same that have been previously established by C2—V3 sequences. Detection of HIV-1 groups M, N, and group O infections with a very high efficiency is feasible by using only one set of primers for all known HIV-1 strains worldwide. Thus, using gp41 region for phylogenetic analysis of HIV-1 strains worldwide provides an alternate and effective tool for determination of distinct HIV-1 variants. In addition, the gp41M/O primer set may have a broad spectrum of future applications, ranging from quantitative measurement of viral load for clinical management of infected patients to utilization as a diagnostic tool for early detection of HIV-1 infection in blood donors. Moreover, the gp41 region has multiple functions ranging from membrane fusion, endocytosis signals, and calmodulin-binding that potentially affect critical cellular signal transduction pathways [12]. These functions along with structural elements that interact with the assembling capsid precursors suggest that this region might play an important role in both virus replication and pathogenesis *in vivo* [12]. Thus, the gp41 protein sequences generated worldwide would provide useful information related to immune response, as well as the information related to T20/DP178 epitope which has recently been used in therapeutic trial to block HIV entry [13]. Finally, the gp41 sequences generated would provide valuable information regarding the diversity and its diagnostic implication within the distinct domains of this HIV-1 multi-functional protein.

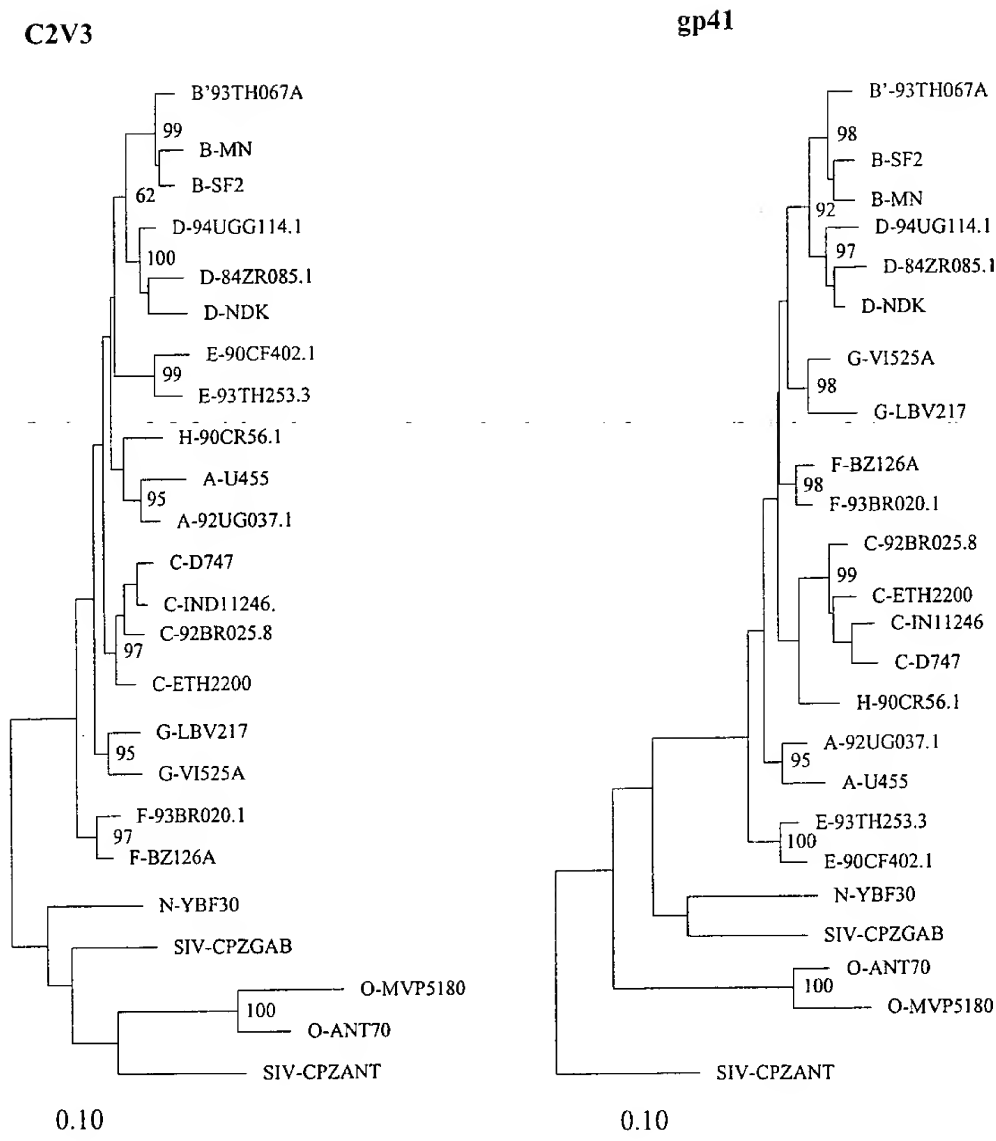


Figure 1. Assignment of HIV-1 reference strains from groups M, N, and O into subtypes by parallel phylogenetic analysis of the *env* C2-V3 and gp41 sequences. After gap stripping, 281 and 354 nucleotide positions were included in the *env* C2-V3 and gp41 alignments, respectively, with a transition/transversion ratio of 1.61 and 1.46. The trees were constructed on the basis of DNA sequences by the maximum-likelihood method using fastDNAmI program (8). HIV-1 subtypes and groups are denoted with prefixes A, B, C, D, E, F, G, and H, and N and O, respectively. SIV-CPZGAB and SIV-CPZANT sequences were included in the analysis. The numbers at the nodes of subtypes A, B, C, D, E, F, H, G, and group O correspond to the bootstrap values in neighbor-joining trees (9) of C2-V3 and gp41. Trees were re-rooted by using HIV-2ROD as an outgroup. The scale bar indicates an evolutionary distance of 0.10 nucleotide per position in the sequence. Vertical distances are for clarity only.

# Phylogenetic Analysis of gp41

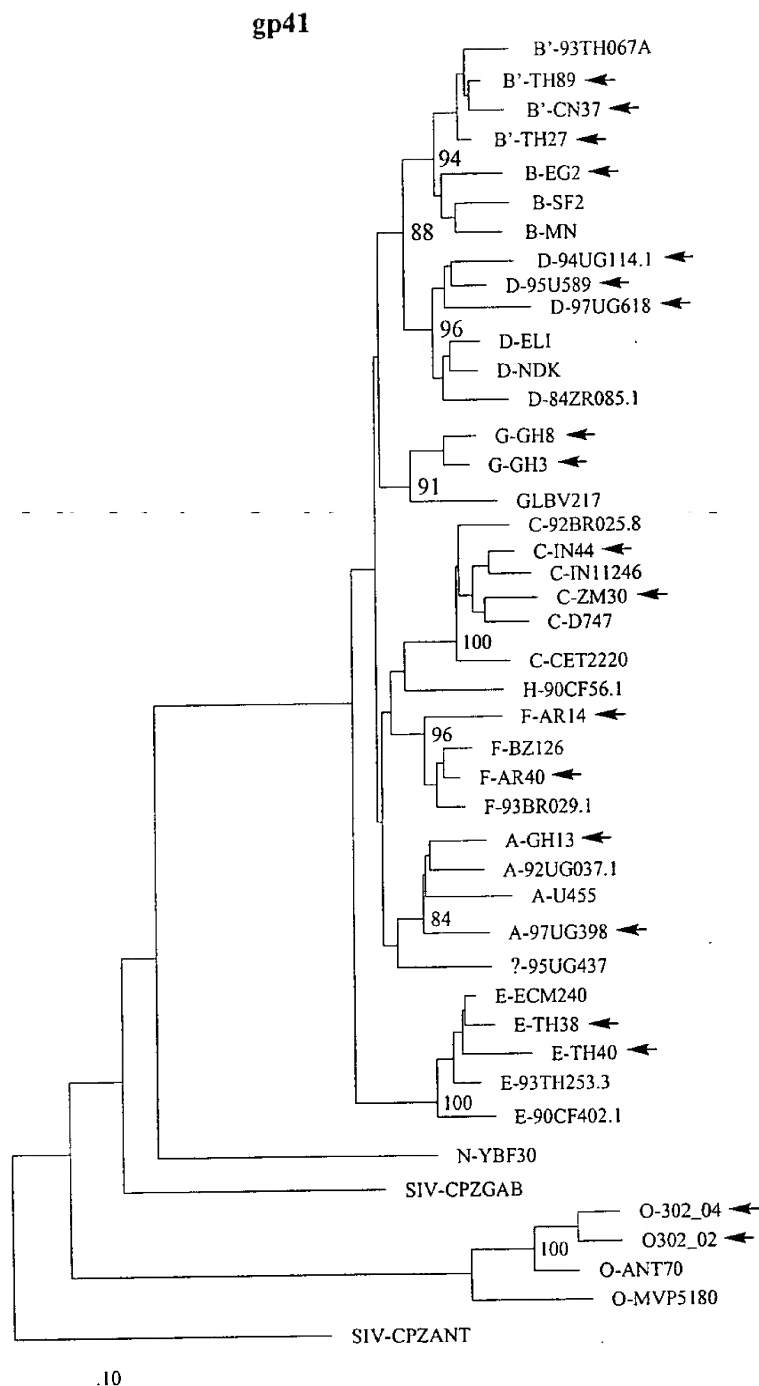
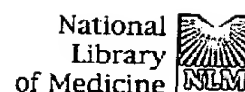
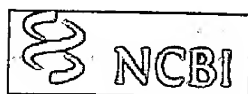


Figure 2. Phylogenetic classification of the new HIV sequences ( ← ) from representative group M subtypes A–G and group O worldwide based on *env* gp41 region. ? represents the unclassified position of sample 95UG437. Genbank accession #AF113577–AF113595. The tree was generated using the neighbor-joining method (9). Numbers at the nodes indicate the percentage of bootstrap values (only values above 80% are shown). The tree was re-rooted by using HIV-2ROD as an outgroup.

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☐ 1: Brain Res Mol Brain Res. 1993 Jan;17(1-2):83-94.

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**Selective in situ hybridization histochemical analyses of alternatively spliced mRNAs encoding beta- and gamma-preprotachykinins in rat central nervous system.**

**Marchand JE, Zaccheo TS, Connelly CS, Kream RM.**

Department of Anesthesiology, Tufts University School of Medicine, Boston MA 02111.




The present study describes the development of an in situ hybridization histochemistry (ISHH) procedure which was employed to selectively monitor cellular distributions of the 2 major alternatively spliced beta- and gamma-species of mRNA encoding preprotachykinin (PPT) molecules found in rat CNS. For these purposes, 2 custom-designed oligodeoxynucleotide probes were synthesized corresponding to complementary sequences of beta- and gamma-PPT mRNAs. In particular, the gamma-selective probe was demonstrated to hybridize to the contiguous regions of RNA flanking the splice site formed by exclusion of exon 4. Initially, Northern blot analyses performed in conjunction with appropriate specificity controls demonstrated selective hybridization of the 32P-labeled beta- and gamma-selective probes to single bands of approximately 1.2-1.3 kilobases in size, consistent with previously established values for rat brain beta- and gamma-PPT mRNAs. In anatomical studies, results obtained from absorptions using competing nonradiolabeled oligonucleotides defined the specificity and selectivity of both probes for targeting their respective species of mRNA immobilized within sections of brain tissue. Extensive ISHH analyses using both beta- and gamma-selective probes demonstrated similar patterns of cellular labeling in all of the examined CNS areas. In addition, data obtained from analyses of adjacent thin sections of the dorsal root ganglia (DRG) indicated that beta- and gamma-PPT mRNAs were colocalized within individual DRG neurons, thereby suggesting generalized coexpression at the cellular level of both forms of mRNA. These data were complemented by semi-quantitative analyses which yielded cellular or intrinsic molar ratios of beta- to gamma-PPT mRNA, of approximately 1:2-1:3, consistent with those values previously determined by nuclease protection analyses. In sum, a reasonable hypothesis evolving from the anatomical studies in combination with previous biochemical data supports the existence of a strong homeostatic mechanism involved in the maintenance of relatively constant intrinsic molar ratios of beta- to gamma-



PPT mRNA by tachykinin-expressing neurons. The biological relevance of this putative fundamental relationship is discussed in the context of posttranslational processing of PPT molecules and of expression of mature tachykinins.

PMID: 8381913 [PubMed - indexed for MEDLINE]

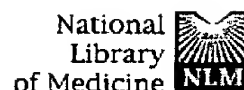
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**(2'-5')-Oligo-3'-deoxynucleotides: selective binding to single-stranded RNA but not DNA.**

**Alul R, Hoke GD.**

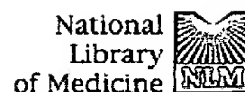
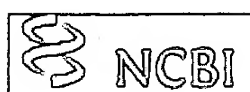
Dyad Pharmaceutical Corporation, Columbia, MD 21046, USA.

Oligodeoxynucleotides with (2'-5') internucleotide linkages have been synthesized on a solid support via standard cyanoethyl phosphoramidite chemistry. This simple change in the oligonucleotide bond connectivity led to unique properties. UV melting temperature experiments indicate that the (2'-5')-oligo-3'-deoxyadenylates, (2'-5')-3'-dA8 and (2'-5')-3'-dA8(s) phosphorothioate, hybridize selectively to single-stranded RNA but not DNA. The complex (2'-5')-3'-dA8:poly (U) (T<sub>m</sub> = 32 degrees C) was nearly as stable as the natural (3'-5')-2'-dA8 and poly (U) (T<sub>m</sub> = 33 degrees C) in 130 mM NaCl, and 10 mM phosphate buffer (pH 7.5). However, no association was observed upon mixing (2'-5')-3'-dA8 and poly (dT). The (2'-5') linkages also confer greater resistance to exo- and endonucleolytic degradation compared with (3'-5')-linked oligomers. The rate of degradation of (2'-5')-3'-dA8 was almost four times less than that of (3'-5')-2'-dA8 in cell culture medium containing 10% heat-inactivated fetal calf serum. An increase in stability for (2'-5')-3'-dA8 against endonuclease activity was observed in both cytoplasmic and nuclear extracts. The nucleic acid selectivity of (2'-5')-oligo-3'-deoxynucleotides may represent an important design feature to improve the efficacy of antisense oligonucleotides.

PMID: 7542046 [PubMed - indexed for MEDLINE]

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**GABAB receptor protein and mRNA distribution in rat spinal cord and dorsal root ganglia.****Towers S, Princivale A, Billinton A, Edmunds M, Bettler B, Urban L, Castro-Lopes J, Bowery NG.**





Department of Pharmacology, Medical School, University of Birmingham, Birmingham, B15 2TT, UK.

The presence of metabotropic receptors for GABA, GABAB, on primary afferent terminals in mammalian spinal cord has been previously reported. In this study we provide further evidence to support this in the rat and show that the GABAB receptor subunits GABAB1 and GABAB2 mRNA and the corresponding subunit proteins are present in the spinal cord and dorsal root ganglion. We also show that the predominant GABAB1 receptor subunit mRNA present in the afferent fibre cell body appears to be the 1a form. In frozen sections of lumbar spinal cord and dorsal root ganglia (DRG) GABAB receptors were labelled with [3H]CGP 62349 or the sections postfixed with paraformaldehyde and subjected to in situ hybridization using oligonucleotides designed to selectively hybridize with the mRNA for GABAB(1a), GABAB(1b) or GABAB2. For immunocytochemistry (ICC), sections were obtained from rats anaesthetized and perfused-fixed with paraformaldehyde. The distribution of binding sites for [3H]CGP 62349 mirrored that previously observed with [3H]GABA at GABAB sites. The density of binding sites was high in the dorsal horn but much lower in the ventral regions. By contrast, the density of mRNA (pan) was more evenly distributed across the laminae of the spinal cord. The density of mRNA detected with the pan probe was high in the DRG and distributed over the neuron cell bodies. This would accord with GABAB receptor protein being formed in the sensory neurons and transported to the primary afferent terminals. Of the GABAB1 mRNA in the DRG, approximately 90% was of the GABAB(1a) form and approximately 10% in the GABAB(1b) form. This would suggest that GABAB(1a) mRNA may be responsible for encoding presynaptic GABAB receptors on primary afferent terminals in a manner similar to that we have previously observed in the cerebellar cortex. GABAB mRNA was also evenly distributed across the spinal cord laminae at densities equivalent to those of GABAB1 in the dorsal horn. GABAB2 mRNA was also

detected to the same degree within the DRG. Immunocytochemical analysis revealed that GABAB(1a), GABAB(1b) and GABAB2 were all present in the spinal cord. GABAB(1a) labelling appeared to be more dense than GABAB(1b) and within the superficial dorsal horn GABAB(1a) was present in the neuropil whereas GABAB(1b) was associated with cell bodies in this region. Both 1a and 1b immunoreactivity was expressed in motor neurons in lamina IX. GABAB2 immunoreactivity was expressed throughout the spinal cord and was evident within the neuropil of the superficial laminae.

PMID: 10998104 [PubMed - indexed for MEDLINE]

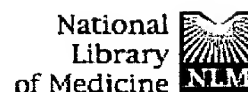
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dITP and Thermus thermophilus DNA polymerase.****Auer T, Sninsky JJ, Gelfand DH, Myers TW.**Program in Core Research, Roche Molecular Systems, Alameda, CA 94501,  
USA.

The ability to selectively amplify RNA in the presence of genomic DNA of analogous sequence is cumbersome and requires implementation of critical controls for genes lacking introns. The convenient approaches of either designing oligonucleotide primers at the splice junction or differentiating the target sequence based on the size difference obtained by the presence of the intron are not possible. Our strategy for the selective amplification of RNA targets is based on the enzymology of a single thermostable DNA polymerase and the ability to modulate the strand separation temperature requirements for PCR amplification. Following reverse transcription of the RNA by recombinant *Thermus thermophilus* DNA polymerase (rTth pol), the resultant RNAxDNA hybrid is digested by the RNase H activity of rTth pol, allowing the PCR primer to hybridize and initiate second-strand cDNA synthesis. Substitution of one or more conventional nucleotides with nucleotide analogs that decrease base stacking interactions and/or hydrogen bonding (e.g. hydroxymethyl dUTP or dITP) during the first- and second-strand cDNA synthesis step reduces the strand separation temperature of the resultant DNAxDNA duplex. Alteration of the thermal cycling parameters of the subsequent PCR amplification, such that the strand separation temperature is below that required for denaturation of genomic duplex DNA composed of standard nucleotides, prevents the genomic DNA from being denatured and therefore amplified.

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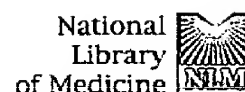
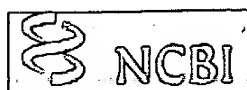
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## Identification and chromosomal localization of a processed pseudogene of human GRK6.

Gagnon AW, Benovic JL.

Department of Pharmacology, Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, PA 19107, USA.

G-protein-coupled receptor kinases (GRKs) phosphorylate agonist-occupied G-protein-coupled receptors, resulting in desensitization of receptor signaling. To date, 6 mammalian GRKs have been identified by molecular cloning. Several lines of evidence indicate that a homologue of GRK6, the most recently described GRK, is present in the human genome. Northern analysis identifies two transcripts which hybridize to GRK6, and genomic Southern analysis indicates that GRK6 is localized to chromosome 5, with a second GRK6-like locus on chromosome 13. To identify the GRK6 homologue on chromosome 13, several sets of closely-spaced primers were designed based on the GRK6 cDNA sequence and then used to amplify human genomic DNA by PCR. Two products were identified, the larger of which is a fragment of the GRK6 gene which contains introns, while the smaller fragment is 94% homologous to GRK6 and contains no introns. In order to further characterize this GRK6 homologue, primers from the 5' and 3' coding regions of GRK6 were used to amplify a product of 1458 base pairs from human genomic DNA. This 1458 base pair PCR fragment displays 94% homology to GRK6 and contains multiple nucleotide insertions and deletions compared to GRK6, including a C to T mutation at base pair 202 which creates a predicted in-frame stop codon. In an effort to determine whether this gene is transcriptionally active, primers designed to preferentially amplify either GRK6 or the homologue were used in reverse transcription PCR. In contrast to the GRK6-specific primers, primers which selectively amplify the GRK6 homologue fail to produce a PCR product in any RNA tested, indicating that this gene is most likely transcriptionally inactive. PCR amplification of rodent/human hybrid cell lines using these same primers confirms the previously established chromosome 5 localization of GRK6, and localizes the homologue to chromosome 13. Northern analysis indicates that the two GRK6-hybridizing species seen in RNA differ by approximately 500 base pairs in the 3' untranslated region, indicating that both transcripts likely arise from differential processing of a single gene. Taken together, these data

indicate that the GRK6-hybridizing species on chromosome 13 is a transcriptionally inactive processed pseudogene of GRK6, while the two GRK6 transcripts differ in the 3' untranslated region.

PMID: 9016947 [PubMed - indexed for MEDLINE]

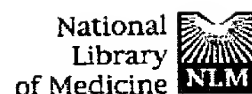
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**A PCR detection method for rapid identification of *Melissococcus pluton* in honeybee larvae.**

**Govan VA, Brozel V, Allsopp MH, Davison S.**

Department of Microbiology, University of the Western Cape, Bellville, South Africa.

*Melissococcus pluton* is the causative agent of European foulbrood, a disease of honeybee larvae. This bacterium is particularly difficult to isolate because of its stringent growth requirements and competition from other bacteria. PCR was used selectively to amplify specific rRNA gene sequences of *M. pluton* from pure culture, from crude cell lysates, and directly from infected bee larvae. The PCR primers were designed from *M. pluton* 16S rRNA sequence data. The PCR products were visualized by agarose gel electrophoresis and confirmed as originating from *M. pluton* by sequencing in both directions. Detection was highly specific, and the probes did not hybridize with DNA from other bacterial species tested. This method enabled the rapid and specific detection and identification of *M. pluton* from pure cultures and infected bee larvae.

PMID: 9572987 [PubMed - indexed for MEDLINE]

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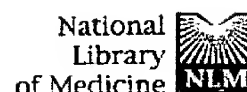
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## A PCR detection method for rapid identification of *Paenibacillus* larvae.

**Govan VA, Allsopp MH, Davison S.**

Department of Microbiology, University of the Western Cape, Bellville 7535 South Africa.

American foulbrood is a disease of larval honeybees (*Apis mellifera*) caused by the bacterium *Paenibacillus* larvae. Over the years attempts have been made to develop a selective medium for the detection of *P. larvae* spores from honey samples. The most successful of these is a semiselective medium containing nalidixic acid and pipermedic acid. Although this medium allows the growth of *P. larvae* and prevents the growth of most other bacterial species, the false-positive colonies that grow on it prevent the rapid confirmation of the presence of *P. larvae*. Here we describe a PCR detection method which can be used on the colonies that grow on this semiselective medium and thereby allows the rapid confirmation of the presence of *P. larvae*. The PCR primers were designed on the basis of the 16S rRNA gene of *P. larvae* and selectively amplify a 973-bp amplicon. The PCR amplicon was confirmed as originating from *P. larvae* by sequencing in both directions. Detection was specific for *P. larvae*, and the primers did not hybridize with DNA from closely related bacterial species.

PMID: 10224028 [PubMed - indexed for MEDLINE]

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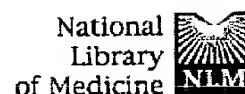
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## PCR-enzyme-linked immunosorbent assay and partial rRNA gene sequencing: a rational approach to identifying mycobacteria.

Patel S, Yates M, Saunders NA.

Virus Reference Division, Central Public Health Laboratory, London, United Kingdom.

A PCR-enzyme-linked immunosorbent assay (ELISA) for amplification and rapid identification of mycobacterial DNA coding for 16S rRNA was developed. The PCR selectively targeted and amplified part of the 16S rRNA gene from all mycobacteria while simultaneously labelling one strand of the amplified product with a 5' fluorescein-labelled primer. The identity of the labelled strand was subsequently determined by hybridization to a panel of mycobacterial species-specific capture probes, which were immobilized via their 5' biotin ends to a streptavidin-coated microtiter plate. Specific hybridization of a 5' fluorescein-labelled strand to a species probe was detected colorimetrically with an anti-fluorescein enzyme conjugate. The assay was able to identify 10 Mycobacterium spp. A probe able to hybridize all Mycobacterium species (All1) was also included. By a heminested PCR, the assay was sensitive enough to detect as little as 10 fg of DNA, which is equivalent to approximately three bacilli. The assay was able to detect and identify mycobacteria directly from sputa. The specificities of the capture probes were assessed by analysis of 60 mycobacterial strains corresponding to 18 species. Probes Avil1, Int1, Kan1, Xen1, Che1, For1, Mal1, Ter1, and Gor were specific. The probe Tbc1 cross-hybridized with the Mycobacterium terrae amplicon. Analysis of 35 strains tested blind resulted in 34 strains being correctly identified. This method could be used for rapid identification of early cultures and may be suitable for the detection and concurrent identification of mycobacteria within clinical specimens.

PMID: 9276419 [PubMed - indexed for MEDLINE]

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